A STUDY ON RECENT ADVANCES IN LABORATORY TECHNIQUES FOR DIAGNOSIS OF MALARIA

Dissertation submitted to

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CERTIFICATE

This is to certify that the dissertation entitled "**A STUDY ON RECENT ADVANCES IN LABORATORY TECHNIQUES FOR DIAGNOSIS OF MALARIA"** is the bonafide original work of **DR.B.SANTHY**, in partial fulfillment of the requirements for **M.D(BRANCH-IV) MICROBIOLOGY** examination of Tamilnadu Dr.MGR Medical University, to be held in September, 2006.

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DECLARATION

I, DR. B. SANTHY, solemnly declare that the dissertation titled "A STUDY ON RECENT ADVANCES IN LABORATORY TECHNIQUES FOR DIAGNOSIS OF MALARIA" is a bonafide work done by me at Government Stanley Medical College & Hospital during 2003 – 2006 under the expert guidance and supervision of Dr.P.R.Thenmozhi Valli, M.D., Professor and Head of the Department, Department of Microbiology.

The dissertation is submitted to the Tamilnadu Dr. M.G.R. Medical University towards partial fulfillment of requirement for the award of **M.D.DEGREE** (BRANCH - IV) in Microbiology.

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INTRODUCTION

"Humanity has but three great enemies fever, famine and war; Of these by far the greatest, by far the most terrible is fever". William Osler[30].

Human malaria is basically a febrile illness caused by species belonging to the genus Plasmodium. Malaria has always been a major public health concern, probably representing the most important parasitic disease in human. Malaria is one of the most common cause for school absentism.

It has been infecting humankind for millennia. Earliest recordings of this disease are available from description of the Chinese and Egyptian civilization between 1700 & 1500 B.C. Recordings can also be found in the Vedic writings of 1600 BC in India. Charaka and Sushrutha gave vivid descriptions of malaria and even associated it with the bite of mosquitoes[56]. Malaria was also a scourge of the western civilization even at the beginning of this century.

Malaria is a protozoan (Coccidiean-Apicomplexa) disease transmitted by the bite of infected female Anopheles mosquitoes, which breed in fresh water[66]. The species that infect humans are four in number. P.falciparum, P.vivax, P.ovale and P.malariae. P.falciparum is predominantly a pathogen of the tropics and produces a more severe form of disease. P.vivax and P. ovale tend to produce infections in more temperate zones that are less severe. Infections induced by P. malariae tend to be more benign.

A heavy burden on tropical communities poses a threat to non-endemic countries and a danger to travellers[30]. Malaria results in loss of economy and productivity. The annual direct and indirect cost of malaria in India has been estimated to be an extent of 0.5 to 1 billion US dollars. Malaria remains uncontrolled to date due to various reasons viz. emergence of drug resistant parasite, pesticide resistant mosquito vector and non availability of suitable and effective malarial vaccine[67].

Presently, the disease malaria seems to be a diagnostic challenge to both the physician and to the laboratories in most of the countries. Hence we need to design and review the current methodology and approach to the diagnosis of malaria in a practical and cost effective way helpful for the patients, the laboratory and for the physician caring for the patient. Concurrently the World Health Organization has begun a dialogue with scientists, clinicians and manufacturers of malaria diagnostic test devices regarding the realistic possibilities for developing accurate and cost effective rapid diagnostic tests for malaria[2].

Malaria rapid diagnostic devices (MRDD) have been developed with the hope that they would offer accurate, reliable, rapid, cheap and easily available alternative to traditional method of malaria diagnosis[15]. Assay for rapid diagnoses have the potential to enhance diagnostic capabilities in those

instances in which skilled microscopy is not readily available[2]. Conventional peripheral blood smear examination for demonstration of malarial parasite remains the "gold standard" for diagnosis of malaria[52]. The Quantitative Buffy Coat (Q.B.C), assay for detection of antigen Histidine rich protein (HRP –2) and Pan Malarial Antigen (PMA) by Immuno chromatographic test (ICT), only HRP –2 antigen by Dot ELISA for P.falciparum and detection of specific nucleic acid sequences (P.f 918 bp, P.v 523 bp) by Polymerase chain reaction (PCR) have been studied here particularly to overcome the disadvantages of conventional peripheral blood smear study.

Rapid, accurate diagnosis is fundamental to effective management and control of malaria. Modern methods of malaria diagnosis include fluorescent microscopy, flow cytometry, automated blood cell analysers, serology-antibody detection, molecular methods and laser desorption mass spectrometry (Hanscheid 1999,Demirev et al 2002)[15], Immunochromatographic assay detect malarial antigen Histidine Rich Protein 2 (HRP-2) and enzymes Parasite Lactate dehydrogenase (pLDH), aldolase and PCR. All these new technologies are compared with accepted "gold standard" method[2].

HISTORY OF THE MALARIAL PARASITE AND ITS DIAGNOSIS

Malaria is caused by the sporozoan parasite belonging to the Genus Plasmodium. Malaria, meaning 'bad air' with its well known symptom of intermittent fever has been recorded in papyri, from ancient Egypt.

5TH CENTURY B.C. : Hippocrates in Greece gave a detailed account of the clinical picture and observed the prevalence of the disease in certain places and seasons[37].

6TH CENTURY A.D. : The relation between the disease and stagnant waters, swamps and marshy lands was recognized and measures to control the disease by effective drainage were practiced in Rome and Greece by 6th century A.D[37].

18TH CENTURY : The name malaria 'mal-bad, aria – air' was given in Italy, as it was believed to be caused by foul emanations from the marshy soil. Disease named "Malaria" by Sir Horace Walpole. 'Paludism, another name for malaria also has a similar origin from palus, Latin for 'marsh'. The recent demonstration of a specific parasitic antigen in Egyptian mummies indicates that malaria was present thousands of year's ago[37].

In 1820 : *Quinine isolated from cinchona bark in Indonesia[61].*

In 1880 : *Alphonse Laveran, a French army surgeon in Algeria, discovered the*

specific causative agent of malaria in the red blood cells of a patient[54,61].

In 1883: *Marchiafava used Methylene blue for staining malarial parasites*[54, 61].

In 1886 : *Golgi in Italy described the asexual development of the parasite in red blood cells (erythrocytic schizogony), which therefore came to be called Golgi cycle*[61].

1886-1890 : *Three different species of malarial parasites infecting man, P.vivax, P. malariae, P. falciparum were described in Italy*[37].

In 1897 : *The mode of transmission of the disease was established when Ronald Ross in secunderabad, India identified the developing stages of malarial parasites in mosquitoes. This led to various measures for the control and possible eradication of malaria by mosquito control. It is tragic that one century down we were still grappling with the problem*[61].

In 1900 : *Observation of mosquito transmission by Ross was supported by Patric manson*[54, 61].

Both Ronald Ross (1902) and Alphonse Laveran (1907) won the Nobel Prize for their discoveries in Malaria[37].

In 1922 : *The fourth species P.ovale was identified*[37].

In 1948 : *Shortt and Garnhalm demonstrated the site of the exo-erythrocytic development in the liver. In the next year, they demonstrated the pre-*

erythrocytic schizogony of P.falciparum[54, 61].

In 1955 : *The World Health organization launched a Worldwide Malaria Eradication Programme. This continued until 1970, when it was official declared a failure*[45, 54].

With the spread of malaria, mosquito control measures were tried. The first large-scale demonstration of this was in Cuba in 1899 and in India in 1935-1939[22]. The World Health Organization between 1953-1958 embarked on the Malaria Eradication Programme[22,45]. But 1972-1978 saw the resurgence of malaria in endemic tropical countries including India.

1965 – 1976 as part of the reorientation of the *Malaria Control* strategy, extensive scientific studies were done on the development of a malarial vaccine (between 1965-1976), which culminated in the production of continuous in-vitro culture technique for *P. falciparum* by *Trager* and *Jensen* in 1976.

National Malaria Control Programme (NMCP) was launched in India in April 1953. It was based on indoor residual spraying with DDT twice a year in endemic areas. Government of India in the Ministry of Health changed the strategy from malaria control to eradication and launched the National Malaria Eradication Programme (NMEP) in 1958. Considering the resurgence of malaria, the Govt. of India in the ministry of Health modified strategy as a Modified Plan of Operation (MPO) to control malaria. The Modified Plan of

Operation under the NMEP came into force from 1st April 1977. In 1999, the Govt. of India decided to drop the term "National Malaria Eradication Programme" and renamed it "National Anti-malaria Programme"[56].

Serological tests - Indirect Fluorescent antibody test (IFAT) & Indirect Haemagglutination Assay (IHA) in study of malaria were developed in early 1960s and there is a sustained interest in this aspect to develop an ideal test which would be effective in the field trials for use in sero-epidemiological surveillance[26].

A test that could be easily interpreted within 5 to 15 minutes of specimen would be a substantial aid to clinicians. Of the numerous new techniques developed for the diagnosis of malaria, Quantitative Buffy Coat (Q.B.C.) technique appears to be a promising test[46].

Other enzymes within the glycolytic pathway of the malarial parasite have been recognized and considered as target for rapid malaria diagnostic tests. Aldolase is a key enzyme in this pathway. Malarial antigens currently targeted by rapid diagnostic test are HRP-2, pLDH and Plasmodium aldolase[2].

PCR based methods are particularly useful for studies on strain variation, mutations and studies of parasite genes involved in drug resistance. The ability to detect and distinguish all species of plasmodium and the enhanced sensitivity make them ideal procedures for the diagnosis of

malaria[2, 17].

The Centers for Disease Control and Prevention recommends that malaria should be considered in the differential diagnosis of febrile patients who have traveled to a region where malaria is endemic and in any patients who experience fevers of unknown origin regardless of their travel history[43, 73].

EPIDEMIOLOGY

Malaria is one of the important parasitic disease of human being affecting 103 countries in the world involving a population of over 2.5billion people and causing 1 to 3 million deaths each year. Around 40% of the World's population is deemed under malaria risk[53]. It is ranked with acute respiratory diseases and diarrhoeal diseases as one of the leading causes of the mortality world wide[18].

GEOGRAPHICAL DISTRIBUTION :

Malaria has been recorded in places as far north as Archangel, Russia and as far south as Cordoba, Argentina and in places as low as the Dead sea (400 meters below sea level) and as high as Cochabamba, Bolivia (2800 meters above sea level). However, malaria is essentially a focal disease and its distribution is patchy in most parts[28].

The relative prevalence of four species of malarial parasites varies in different geographical regions. *P. vivax* is the most widely distributed, being common in Asia, North Africa and Central and South America. *P. falciparum,* the predominant species in Africa, Papua New Guinea and Haiti, is rapidly spreading in South East Asia and India. *P. malariae* is present in most places but is rare except in Africa. *P. ovale* is virtually confined to West Africa where it ranks second after *P. falciparum.* Malaria may occur in endemic as well as epidemic patterns[40].

WORLD WIDE DISTRIBUTION : At the end of 2004, 107 countries and territories had areas at the risk of malaria transmission. Some 3.2 billion people lived in areas at risk of malaria transmission. An estimated 350 to 500 million clinical malaria episodes occur annually; most of these are caused by infection with P.falciparum and P.vivax[75].

MALARIA IN INDIA : 65% of malaria in India is caused by P.vivax and 35% by P.falciparum. Nearly 0.98 million cases of malaria were reported during the year 2001 of which nearly 0.46 million were falciparum cases. Highly endemic areas for malaria include the north – eastern region and tribal forested and hilly areas of several states including Maharashtra, and selected non-tribal districts. Nationwide, the reported incidence of laboratory confirmed cases has declined from 3.0 million in 1996, 2.1 million in 2001 and 1.78 million in 2003[75].

IN TAMILNADU : 43,604 cases were reported in 2003[75], Chennai in the year 2004, 28,224 cases were reported. (Courtesy of Chennai Corporation).

TRANSMISSION : Man is the only source of malarial infection. Infected man suffering from acute and chronic malaria and carriers harbouring gametocytes are the reservoirs of malaria[55]. The human beings are the intermediate host; the female Anopheles mosquito is the definite host (vector). Malaria is transmitted by the bite of infected female Anopheles mosquitoes. This is the major mode of

transmission. But unnatural methods of transmission as a result of blood transfusion when the donor harbours malarial parasite (Bruce Chwatt 1974, 1980) drug addiction and congenital infection of the new born from an infected mother (Bruce chwatt 1980) have also been reported in the first half of this century[28].

AGENT:

PLASMODIA : Malarial parasites belong to *Phylum Apicomplexa; Class* Telosporea, *Order* Eucoccidia, Suborder Haemosporina and Genus Plasmodium. Of the 120 existing species of Plasmodia, only four are the primary agents of human malaria- P.falciparum, P.vivax, P. malariae & P.ovale (Phillips 1983)[23].

The malarial parasite passes its life cycle in two different hosts. Man being the intermediate host allows the asexual cycle to continue. This cycle consists of primary-erythrocytic schizogony, erythrocytic schizogony, and gametogony and in P. vivax alone the secondary exoerythrocytic schizogony. The duration of each phase varies with the species.

In Mosquitoes : The female Anopheles mosquito forms the definitive host. This cycle is initiated by the mosquito's ingestion of the sexual forms (male and female gametocytes) developed in the human host, during the blood meal. Within the mosquito further development occurs with the formation of

sporozoites, which are infective to man[23].

Vector : only female Anopheles mosquitoes can transmit human malaria. The male Anopheles feeds exclusively on nectar and fruit juices while the female feeds on warm blooded animals. There are about 422 species of Anopheles mosquitoes through out the world, but only some 70 species are vectors of malaria under natural conditions[28].

In India the predominant vector is A. culifaciens though A. fluviatilis, A. stephensi, A. sundaicus, A. minimus and A. philippinensis also exist (Bruce Chwatt 1980). The vectors of major importance are A. culifaciens in rural areas A. stephensi in urban areas; Breed in fresh water; Anopheles mosquitoes have nocturnal feeding habit, between dusk and dawn[28].

Environment : Variations in climatic conditions has a profound effect on the mosquito and the malarial parasite. A temperature of 18^{o}C – 30^{o}C and humidity of 60%-70% are optimal for the vector and the parasite. Rainfall is also important by increasing breeding sites and humidity (WHO 1979, Bruce Chwatt 1980)[28].

Spleen palpation and peripheral blood examination are important methods in malaria surveillance and epidemiologic studies on prevalence of the disease[29,63]. The use of spleen rate as a malariometric index has the

disadvantages that in areas where antimalarial measures have been instituted. The spleen rate classification of endemicity is not applicable (Bruce Chwatt 1980) and the splenomegaly especially in tropical regions is a non-specific sign, which may be due to causes other than malaria (Kagan 1972).

- During the past few years, there has been an increased awareness of the importance of trained and qualified personnel to perform diagnostic procedure.

- These results obtained by intensive basic research are currently leading to improved diagnostic procedure.

- The rapid development of new methods and techniques in the area of molecular biology has gained new insights into the genetic and structural features of malarial parasite.

PATHOGENESIS AND PATHOLOGY OF MALARIA

The female Anopheline mosquito becomes infected when it feeds on human blood containing gametocytes, the sexual forms of the malarial parasites. Development in the mosquito takes from 7-20 days. The infection starts with the bite of an infected mosquito, when the sporozoite is introduced in to the skin together with mosquito saliva. The sporozoites rapidly make its way to the liver, where it invades a hepatocyte. Within the hepatocyte the parasite undergoes a period of differentiation and multiplication to produce the pre or exoerythrocytic schizont, containing a few thousand merozoites. Rupture of the schizont releases more merozoites into the blood and invade erythrocytes where they develop through ring forms to trophozoites and eventually

schizonts. P.vivax invades the youngest red cells where as P. falciparum invades red cells of any age[16].

The pre-erythrocytic stage of infection produces minimal histopathological changes and absolutely no detectable symptoms or functional disturbances in the host. Pathological process in malaria is the result of the erythrocytic cycle. In case of P. vivax and P. ovale infections, arrested development of some sporozoites in liver cells results in dormant forms, hypnozoites from which the infection may later relapse. P. falciparum and P. malariae have no persistent exoerythrocytic phase but recrudescences of fever may result from multiplication of parasites in the red cells which have not been eliminated by treatment and immune processes.

In case of P.falciparum the following changes that occur to the infected erythrocyte are altered membrane transport mechanisms, development of protuberances or knobs beneath the surface membrane, expression of various surface neoantigens, development of cyto-adherent and rosetting properties and digestion of haemoglobin to pigment. The secondary effect of these changes are related to the host's immunological response to parasite antigens and altered red cell surface membranes such as the stimulation of the reticuloendothelial system, changes in regional blood flow and vascular endothelium and systemic complications of altered biochemistry, anaemia, tissue and organ hypoxia and cytokine production[21].

The fever, febrile paroxysms, headache, a variety of aches and pains and prostration, the most familiar and consistent symptoms of an acute malaria attacks, are probably the result of cytokines released from macrophages at the time of schizont rupture. The malarial toxin released at schizont rupture is the lipid, glycosyl phosphatidyl inositol (GPI) anchor of a parasite membrane protein (MSP1). Cytokines such as tumor necrosis factor (TNF), and interleukin-1 (IL-1) have been implicated as the cause of malarial fever when the mature schizont ruptures releasing red cell fragments, merozoites, malarial pigment and other parasitic debris. Macrophages and polymorphs phagocytose these and release large quantities of endogenous pyrogens leading to elevation of temperature[21].

Pathology : The pathology in malaria is due to haemolysis of infected red cells and adherence of infected red blood cells to capillaries. Malaria is always accompanied by haemolysis and in a severe or prolonged attack anaemia may be profound. Typical pathological changes are seen primarily in the spleen, liver, bone marrow, lungs, kidney and brain.

Chloroquine resistant P. falciparum was first reported in Columbia and Thailand in the late 1950s[55]. Several mechanisms can account for changes in drug sensitivity in the malarial parasite.

- Physiological adaptation due to non-genetic changes, selection

of previously existing drug resistant cells from a mixed population under drug pressure.

- Spontaneous mutation.
- Mutation of extranuclear genes
- Or the existence of plasmid like factors [20]

IMMUNITY

Host immunity in malaria is of two types-

-Natural or innate immunity

-Acquired immunity

Innate Immunity: Innate resistance is seen in certain population e.g. person with red cells negative for Duffy blood group determinants are resistant to P. vivax infections (Phillips 1983). Young babies have a reduced susceptibility especially to P. falciparum, probably due to maternal antimalarial antibodies and also the presence of haemoglobin F which is less suitable for the parasite's existence and multiplication (Phillips 1983). Glucose -6- phosphate dehydrogenase deficient individuals are protected against malaria. This enzyme is necessary for the respiratory process of the parasite[52].

P. falciparum does not multiply in sickle red cells containing abnormal haemoglobin S[38]. Severe malnutrition and iron deficiency may confer some protection against malaria. During severe famine in North Africa, malaria was rare but on providing food and iron supplements the patients began to develop malaria. Spleen plays an important role in immunity against malaria. Splenectomy enhances susceptibility to malaria[62].

Falciparum malaria is more severe in pregnancy particularly in primigravida, and may be enhanced by iron supplementation. HLA-B 53 is

associated with protection from malaria[38].

Acquired Immunity : Both humoral and cellular immunity play a protective role in malaria.

Humoral Component : Where malaria is hyperendemic, the acquisition of clinically evident protective immunity in young children is associated with significant increase in serum gamma - globulin and a greater increase is seen in adults (McGregor 1981). Of the increased gamma globulins, only 5-10% are associated with specific action against the malarial parasite (curtain et al 1964)[49].

Cellular Immunity : Involvement of T. lymphocytes in malaria has been shown experimentally (Kass et al 1971, Cohen et al 1974, Greenwood 1974, Allison 1981). However it has been postulated T cells do not function alone, but co-operate B cells for induction of specific antibody (McGregor 1981)[69].

CLINICAL MANIFESTATIONS

The main clinical features are fever peaks with rigor followed by anaemia and splenomegaly. The clinical features of malaria vary from mild to severe and complicated according to the species of the parasite present, the patient's state of immunity, the intensity of infection and also the presence of concomitant condition such as malnutrition.

Prodromal period : Malarial paroxysm is preceded by a prodromal period, which varies from a few to several days. Non-specific symptoms such as malaise, myalgia, headache and fatigue are usually seen during the prodromal period[3.11.60].

Malarial paroxysm : Occur with definite intermittent periodicity depending upon the species of the parasite involved. Comprises of three successive stages cold, hot and sweating stage[4,13,71].

Cold Stage : Lasting for 15-60 minutes; the patient experience intense cold and shivering[4,13,71].

Hot Stage : Lasting for 2-6 hrs, when the patient feels, intense heat. Patient develops high fever (40-40.6^{o}C), severe headache, nausea and vomiting[4,13,71].

Sweating stage : Patient is drenched in profuse sweat. The temperature drops rapidly and patient usually falls into deep sleep, to wake up refreshed.

The paroxysm usually begins in the early afternoon and lasts for 8-12 hrs. The periodicity of the attack varies with the species of the infecting parasite (P.v. 48 hours, P.f. 48 hours., P.o. 48-50 hours, P.m. 72 hours)[4,13,71].

All clinical manifestations in malaria are due to products of erythrocytic schizogony and host reactions to them. The exoerythrocytic liver cycle and gametogony do not appear to contribute to clinical illness[4].

Anaemia : Anaemia is normochromic and normocytic. It is severe in falciparum malaria. The pathogenesis of anaemia appears to be multifactorial. These include.

- The lysis of parasitized and non-parasitized red blood cells.
- The suppression of erythropoiesis in the bone marrow.
- Increased clearance of non-parasitized RBC's by the spleen.

Hepatosplenomegaly : Physical examination in uncomplicated malaria shows moderate splenomegaly and tender hepatomegaly or may even reveal no abnormalities. Splenomegaly due to massive proliferation of macrophages, phagocytize both parasitised and non-parasitised red blood cells. Lymphadenopathy does not occur in malaria[4].

P. falciparum is the most pathogenic malaria species.

Factors contributing to virulence of P. falciparum :

High level of parasitaemia : In P. falciparum malaria, parasite density exceeds more than 250,000-300,000 / ml of blood. Nearly 30% - 40% of total body red cells are parasitized. This is caused by invasion of erythrocytes of all ages (young and old) by P. falciparum. A level of 25% parasitaemia is usually fatal.

Sequestration of parasite: Sequestration means a condition of holding back of mature parasites in vital organs. This phenomenon is shown exclusively by P. falciparum and is due to its ability to cytoadhere[4,13]. Once inside the erythrocytes, P. falciparum merozoites produce a protein within erythrocyte surface membrane in form of a deformation called knobs. These knobs produce high molecular weight, strain specific adhesive protein. These proteins in turn mediate attachment of parasites to receptors on the endothelium of capillaries and venules. This causes sequestration of parasitized erythrocytes in the small post capillary venules of the internal vital organs specifically, the CNS, kidneys, spleen and lungs[4,13].

Gametocyte – infected erythrocytes do not have any knobs, so do not stick to the venular endothelium, hence are not sequestrated, and therefore are demonstrated in the peripheral blood smears. This phenomenon of sequestration does not occur in any other malarial parasites.

Cytokines : P. falciparum produces a number of cytokines such as IL-1,

TNF and IFNγ. These cytokines act specifically on a number of receptors present on the surface of endothelial cells in the small capillaries and post capillary venules[49].

P. vivax malaria may not initially present with the classical symptoms, due to the release of non-synchronous broods of parasites. Herpes labialis is common in vivax malaria. Cerebral irritation, polyuria and in children fulminant infections and severe anaemia have been reported (Bruce Chwatt 1980). Vivax malaria is important not due to its fatality but due to the debility it can produce by its relapses (Bruce Chwatt 1980).

When more than 3% - 5% of erythrocytes are parasitized, the condition is known as severe falciparum malaria Complications of severe falciparum malaria are[4,13].

- Pernicious malaria
- Black water fever
- Tropical splenomegaly
- Pulmonary oedema
- Renal failure
- Hypoglycaemia with lactic acidosis

DIAGNOSIS OF MALARIA

The diagnosis of malaria can be subdivided into clinical and laboratory which include parasitological, serological and molecular biological detection[70].

Importance of diagnostic methods in malaria:

- Diagnosis of individual cases.
- Prognosis of cases.
- Monitoring drug resistance.
- Epidemiological studies.

Clinical Diagnosis : The condition is considered in any person who has a febrile illness and who has come from the area endemic for malaria, received blood transfusion or used intravenous drugs[55].

Laboratory diagnosis :

Specimen : Peripheral blood : Under strict aseptic precautions blood obtained by pricking a finger or earlobe is the ideal sample because the density of developed trophozoites or schizonts is greater in blood from this capillary rich area[2].

Venous blood : Under strict aseptic precautions blood obtained by venepuncture collected in presterilized aliquot with anticoagulant heparin or sequestrine (EDTA) is acceptable for making smear if used shortly after being drawn to prevent alteration in the morphology of white blood cells and

malarial parasite. Blood should be taken at any time over the course of infection before starting antimalarial treatment, but the best time is midway between paroxysm of chills and fever when the greatest number of intracellular organisms are present[55].

Parasitic diagnosis : Parasitic diagnosis depends on the demonstration of parasite in the blood.

Methods of examination :

1. Light Microscopy – Thick & Thin peripheral smear
2. Fluorescence microscopy :
    - Quantitative Buffy Coat (Q.B.C.)
    - Acridine Orange Staining (A.O.)
    - Benzo thio Carboxy Purine Staining (B.C.P.)
3. Micro Concentration technique

Light Microscopy : Conventional light microscopy of stained blood smear is the “gold standard” for confirmation of malaria. Both thick and thin smears are prepared from the capillary blood. They are stained with one of the Romanowsky’s stains such as Leishman’s’, Fields’, Wrights’, Giemsa’s or Jaswantsingh Bhattacharjee stain[55].

Thick Smear : The thick blood film concentrates the layer of red blood cells

on a small surface by a factor of 20 to 30 and is stained as an unfixed preparation. The thick blood film provides enhanced sensitivity of the blood film technique and is much better than the thin film for detection of low levels of parasitaemia and reappearance of circulating parasites during infection recrudescence or relapse. Atleast 100-200 fields, each containing 20 WBC, should be examined before a thick smear is reported as negative for malaria[2]. It is not used for species diagnosis.

Thin Smear : Because of the fixed monolayer of RBC available in this procedure, the morphological identification of the parasite to the species level is much easier and provides greater specificity than the thick-film examination. The thin film is often preferred for routine estimation of parasitaemia because the organisms are easier to see and count. The ability to count parasites in sequential blood films enables the response to therapy to be monitored, particularly for *P. falciparum* infection[2, 31].

The diagnosis of malaria is ruled out by obtaining negative thick blood smears on atleast three different occasions. (12-24 hrs; 3 subsequent days) Light Microscopy is a sensitive method. In the hand of a skilled microscopist 5-10 parasites / µl of blood can be detected by microscopy. Microscopy is the most inexpensive test to confirm malaria[55].

Although more stable stains are now available, the staining process may

take up to 60 minutes of preparation time to produce a stained thin or thick film and is labor-intensive. Interpretation requires considerable expertise, particularly at low levels of parasitaemia. In addition patients with P. falciparum malaria may have parasites sequestrated in the deep capillaries (Spleen, liver, bone marrow), and organisms may not be seen in equivalent numbers in circulating peripheral blood obtained by venepuncture or fingerstick. Thus a P. falciparum infection may easily be missed because there are insufficient numbers of parasites for detection in the blood films. Post treatment examination of blood film can result in the observation of a number of circulating parasites; however, the organisms may be the non-viable parasites yet to be cleared by host, thus prolonging the assumed positivity of the patient and extending treatment[2].

Chinese workers recommend that intradermal smear to be taken from multiple punctures on the upper forearm using a 25-gauge needle. The puncture should not bleed, but a serosanguinous fluid can be expressed on to a slide by squeezing. This method is said to have a better sensitivity for diagnosing as this can pick up cases in which the blood smear may be negative due to peripheral sequestration[39].

Q.B.C. Assay : In 1976, Stephen Wardlaw and Robert Levine invented the Q.B.C., both of them expanding the utility of Q.B.C. as a general haematological tool for humans and animals and a means of diagnosing

haemoparasitic diseases. This is a sensitive method for detection of malarial parasites. It is based on ability of acridine orange to stain nucleic acid containing parasites and leucocytes take up the dye, which is fluorescent when examined under a fluorescent microscope. Acridine-orange stained malarial parasites appear brilliant green. This elegant method is easy to perform, easily interpreted within 5-15 minutes. Q.B.C. is more sensitive method than the thick blood smear. It can detect as low as 3-4 parasites /µl, of blood [7,46,55,59,70] Q.B.C. assay has advantages beyond any doubt in terms of speed, ease, reliability and sensitivity in diagnosis of malaria. In addition it has been of immense help in identifying the infective carriers with a very low parasite burden. Macroscopic examination of QBC tube often enables to predict a positivity of malaria. It has also been noted that colour of plasma in buffy coat also indicates about anaemia[59].

QBC assay has also been helpful in detection of microfilaria after Hetrazan provocative test and / or night blood examination ; Other uses are the diagnosis of relapsing fever and to study the haematological parameters (e.g.) hamatocrit platelet count, WBC count etc.,[59]

Disadvantage : Cost
Species identification is not possible

A.O. Staining method : In this method blood smears are prepared on a slide and are stained with acridine orange. This results in a differential staining of the malarial parasites. Nuclear DNA is stained green and cytoplasmic RNA

red, and aids in the identification of the malarial parasite. The stained slide is examined either with a fluorescence microscope or a microscope equipped with an interference filter system (Kawamoto1991), allows quicker screening of films, because parasites are more readily recognized and a lower power lens may be used.[42,44,46,51,55,70].

Benzothio carboxy purine (BCP) staining : BCP can be applied directly to a lysed blood suspension or to an unfixed but dry thick blood film and intensely stains the nucleic acid of parasites after penetrating RBCs. The dye does not stain nuclei of WBCs. This is useful in field laboratory for mass screening because of rapid staining and evaluation but it requires a fluorescent microscope[2,10].

With experience, workers using methods involving fluorochrome compounds are able to detect parasites rapidly and accurately. However, an important limitation of method, involving both A.O. and BCP is their inability to easily differentiate among Plasmodium species[2].

Microconcentration technique: Blood is collected in microhaematocrit tube and centrifuged at high speed. The sediment is mixed with normal serum and smear prepared. Although, the positivity rate is increased by this technique, but there is change in morphology of the parasite[10].

Serodiagnosis :

Immunological methods provide the means for detecting either the parasite antigens or the host antibodies directed against the parasite. The detection of antigens may be an acceptable alternative to parasite detection, particularly if the assay is robust, inexpensive, easy to use in field conditions and does not require a microscope, but the detection of antibodies merely provides information on past malaria experience and is of limited use for individual diagnosis[70].

Serological tests commonly used for detection and measurement of malarial antibodies[26] :

Test	**Current application**
Immuno precipitation	Epidemiological studies and research
Indirect fluorescent antibody test (IFAT)	Epidemiological studies, research and aids to diagnosis
Indirect Haemagglutination (IHA)	Epidemiological surveys
Enzyme Linked Immunosorbent Assay	Epidemiological studies and aid to diagnosis
Radio immuno assay	Research
Merozoite inhibition in culture	Research

Serology cannot match the sensitivity of microscopic detection of malarial parasite, but may assist diagnosis of light infection. It has got limited value in clinical diagnosis because they will not differentiate between an active and a past infection[10]. Serological methods of diagnosis of malaria have become of practical value since 1962 when the Indirect fluorescent antibody test (IFAT) was introduced. Serological tests are of value in providing a retrospective confirmation of malaria infection and are also used for

epidemiological purpose when parasitaemia is low. Other applications of serological techniques are the diagnosis of hyperactive malaria splenomegaly and the screening of blood collected for blood banks. It should be remembered however that these tests are of limited value in diagnosis of acute phase of diseases in high prevalence areas[26].

ANTIGEN DETECTION :

In contrast to antibody detection, where blood as well as plasma and serum can be used, a positive antigen detection assay is most useful in detection of current infection. The ideal target antigen

- It should not persist after parasitaemia disappears.
- Should be abundant in the blood (or other bodily fluids such as urine) to maximize sensitivity.
- Should be highly specific to malaria without any cross reactions[70].

Tests for detecting malarial antigens are based on either an antigen capture or an antigen competition format and often use ELISA or the Radio immuno assay (RIA) methodology. Once optimal reagents have been identified (i.e. monoclonal or polyclonal antibodies to specific malarial antigens) the assay may be simplified using a simple agglutination or dipstick method. The best antigen detection assay described have a maximum sensitivity of 0.01-0.001% parasitaemia. (Mackey et al 1982. Fortier et al Khushmith et al 1987, Taylor and Voller 1993)[70].

Malarial antigens currently targeted by rapid diagnostic test (RDT) are HRP-2, pLDH and Plasmodium aldolase.

Histidine rich protein : (HRP)

P. falciparum infected RBC's synthesize three histidine-rich proteins, HRP-1, HRP-2 & HRP3. HRP-1 was identified in all knob-positive P. falciparum parasites. HRP-2 was identified in all P. falciparum parasites regardless of the knob phenotype and was recovered from culture supernatants as a secreted water-soluble protein. HRP-3 was present at the lowest abundance compared to HRP-1 & HRP-2. Neither HRP-1 nor HRP-2 was found in a range of other knob-positive and negative strains of non-P.falciparum malaria. HRP-2 is a water soluble protein produced by asexual stages and young gametocytes of P. falciparum. It is expressed on the RBC membrane surface and because of its abundance in P. falciparum it was the first antigen to be used to develop on RDT for its detection[2].

pLDH and aldolase

pLDH an enzyme found in the glycolytic pathway of the malarial parasite is produced by sexual and asexual stages of the parasite. Different isomers of pLDH for each of the four Plasmodium species infecting humans exist, and their detection constitutes a second approach to RDT development. Several other enzymes of the malarial parasite glycolytic pathway, notably

aldolase have been suggested as target antigen for RDT for species other than P. falciparum[2].

Enzyme parasite lactate dehydrogenase (pLDH) detection had been used to monitor in vitro drug susceptibility assay (Makler and Hinrichs 1993). Now it has also become useful for detection of Plasmodium parasitaemia (Knobloch and Henk 1995). The principle of this assay is that pLDH has different biochemical characteristics from human LDH and may therefore be differentially measured using a simple colorimetric assay. This assay can be used for both quantitative and qualitative estimation of parasitaemia; Can detect as low as 0.1% parasitaemia; not species specific. Now the sensitivity of the assay has been currently improved, by combining it with an antigen capture method using a monoclonal antibody to pLDH; this improves sensitivity 100 times (Piper et al 1995)[70].

RDT dipstick format kits for the detection of malarial antigens are now commercially available. Many field and laboratory studies have been completed that compare immunochromatographic methods with results obtained by conventional microscopy, fluorescence microscopy and PCR[2].

Assay for detection of HRP-2

Three commercial dipstick formats for HRP-2 detection are

- Parasight-F
- ICT Pf
- PATH FALCIPARUM, Malaria IC.

Parasight F (Shiff, Premij and Minjas 1993) is a commercial detection test in dipstick format in which a monoclonal antibody captures, a specific antigen of P. falciparum (HRP2, a molecule present in the parasite throughout the erythrocytic cycle) if antigen is present a positive result is indicated by a visible line on the dipstick[12,32,70].

Advantage :

1. Rapid and Simple
2. Takes only 10 minutes.

This simple, robust assay requires no additional equipments or trained personal; Can be used in field surveillance.

Disadvantage :

1. Cost
2. Quantitation is not possible[12,70].

Since HRP-2 is expressed only by P. falciparum these tests will give negative results with samples containing only P. vivax, P. ovale or P. malariae; many cases of non-falciparum malaria may therefore be misdiagnosed as

malaria negative. HRP-2 antigen has been shown to persist and is detectable after the clinical symptoms of malaria have disappeared and parasites have apparently been cleared from the host[2].

Assay for detection of pLDH

The commercial dipstick format for pLDH detection is OptiMAL-IT assay. This assay is based on the detection of intact and functional pLDH specific for P. falciparum and pan-pLDH (PMA) an antigen that is common to all four species of malaria in whole blood. The test uses two specific monoclonal antibodies that have been immobilized across the test strip[2].

The OptiMAL rapid malaria test is a patient point of care immunochromatographic test that can be performed with a drop of finger stick blood. The entire process takes approximately 15 minutes and results are visually interpreted. The OptiMAL dipstick compared well with blood film microscopy for monitoring antimalarial treatment and could be a useful replacement for microscopy to monitor treatment in places where facilities for microscopy are either lacking or inadequate. In developed countries it could be a useful adjunct to blood film microscopy and it permits a reduction in duration of hospitalization and give an early warning of treatment failure[31, 68].

The ICT Malaria P.f/P.v.: This is a rapid in vitro immunodiagnostic test for the detection of circulating P. falciparum HRP-2 and an antigen

common to all four species of malaria, pan malarial antigen (PMA) in whole blood. The test uses two specific monoclonal antibodies that have been immobilized across the test strip. The results are interpreted within 10 minutes[19, 31, 35, 74]. A limitation of this test is that it cannot speciate P. falciparum mixed infections.

DOT ELISA FOR P. falciparum : Polyclonal antibodies to HRP-2 antigen are coated to the Nitrocellulose membrane. Antigen present in plasma / serum binds to this antibody. A revealing antibody with high sensitivity and specificity is added. The resulting complex is detected using enzyme substrate reaction. Appearance of brown coloured dots on 2 NC tips indicates positive result.

- This kit detects the P. falciparum specific HRP-2 antigen.
- No specialized training required for use of the kit.

Urinary antigen in Malaria :

- Rodriguez del et al (1991) first demonstrated that diagnosis of falciparum malaria can be made by detection of specific antigen in the urine.

- Detection of urinary antigen offers non-invasive, easy and safe alternate means of diagnosis of parasitic infections not only in the field conditions but also in routine clinical laboratories.

- The main advantage of testing the urine is that unlike blood and serum, which need to be collected by an invasive procedure requiring technical expertise and disposable syringes, the collection of urine is a non-invasive procedure.

- The urine specimens can be collected easily and frequently without causing any inconvenience to the patient.

- This would make the urine as a specimen of choice, particularly in children, elderly and those unwilling to give their blood for investigation[47].

Molecular Technique :

Molecular metholds based on DNA amplification have been applied to malaria diagnosis since the late 1980s. Their value lies in their high sensitivity, detecting ≤ 5 parasites / μl. With nested or seminested PCR methods targeting the small – subunit 18S r RNA gene, all four species could be identified. PCR also showed increased sensitivity in comparison to microscopy for the diagnosis of mixed infection. Unfortunately, these conventional PCR assay are technically demanding and time consuming. Moreover, they are prone to carry over contamination during the manipulation of post amplification products, a problem already observed in the unique study testing the use of PCR as a routine method for malaria diagnosis[50].

Real time PCR methods are a recent development of established PCR methodologies. They use fluorescent labels for continuous monitoring of amplicon formation throughout the reaction. The advantages of these techniques are quantification of pathogen, reduced risk of contamination and availability of results within 3 hours (including sample preparation)[50,58].

PCR

- Highly sensitive technique
- Highly species specific
- Can detect parasites in dried blood specimens
- Detect drug resistant in P. falciparum
- A single P. falciparum can be detected in 20 µl of blood using PBRK-1 primer, 100 times more sensitive than of thick smear[55].

The application of DNA or RNA hybridization to malaria diagnosis has several advantages over traditional methods. Although the methodology is never likely to be useful at the peripheral level of health care in its present form, it may have a place as a research tool to monitor malaria control programmes, to perform quality control checks on microscopic diagnoses or to determine the distribution of important genes (e.g. genes associated with drug resistance).

In these methods, a known sequence of nucleic acid (oligonucleotide) is

synthesized and labeled either with radioactive 32_p or a non-radioactive colorimetric reagent and this 'probe' is used to detect parasite nucleic acid, taking advantage of the fact that complementary sequences will hybridize.

The simplest version of this technique is the use of DNA probes to detect parasites directly in a drop of patient's blood immobilized on a filter paper (Franzen et al 1984); in this test format, the sensitivity and specificity of the technique expends largely upon the choice of nucleic acid sequence. Species-specific small subunit ribosomal RNA sequence help to differentiate between P. falciparum, P. vivax, P. ovale, or P. malariae (snoop et al 1993)[70]

DNA probe : - Highly sensitive

- Detect low parasitaemia
- Useful to detect drug resistance.

Recent improvement in molecular technologies like nucleic acid hybridization and PCR has come to the diagnostic field with immense promises. Nucleic acid probes to either DNA or RNA have shown to be highly specific for target sequences of all the four species of human plasmodium. PCR method because of its high sensitivity has been utilized for screening of blood donors to exclude transfusion malaria. PCR assay has also been utilized for epidemiological study of malaria[7].

CULTURE :

Culture is not used for routine diagnosis of malaria. Bass and Johns (1912) reported for the first time the in vitro cultivation of malaria parasites (P. falciparum and P. vivax). In 1976, Trager and Jensen successfully cultivated and maintained P. falciparum in vitro in human red blood cells. In this procedure, culture medium consisted of RPMI 1640 medium, a thin layer of stationary human blood cells and an overlay of medium containing human serum, maintained under an atmosphere with 7% Co_2 and 1% - 5% O_2. Recently various modifications of the culture media have been described. Rabbit serum or bovine serum supplemented with hypoxanthine has been found to support parasite growth, though at a reduced rate. P. falciparum now remains the only parasite widely cultivated in its asexual intra-erythrocytic stage.

Invitro culture of malarial parasites at different development stages is essential for

- Studies on the mechanism of invasion of erythrocytes by merozoites.
- Analysis of parasite antigen and the isolation of protective antigens for the development of vaccines, and as source of antigen for sero-epidemiological studies.
- Bio-chemical and metabolic studies including stage specific changes and
- The study of sensitivity or resistance of parasites to various drugs[55].

Treatment :

Treatment of malaria is primarily based on

- Specific antimalarial chemotherapy
- Supportive therapy.

Antimalarials inhibit growth of malarial parasites by increasing internal pH of parasites by concentrating within acid vesicles of parasite. They also inhibit utilisation of haemoglobin and metabolism of parasite. Chloroquine, Quinine, Quinidine, Mefloquine, halofantrine and artemisinin compounds are the rapidly acting drugs for treatment of acute malaria[55].

Chloroquine is the drug of choice for drug sensitive P. falciparum. It is used for treatment of cases with acute uncomplicated P. falciparum malaria and for prophylaxis. A combination of sulphadoxine and pyrimethamine (fansidar) was used for treatment of chloroquine resistant P. falciparum. Fansidar resistant P. falciparum has also been reported in the early 1980s. Mefloquine is a blood schizonticide. It is used for prophylaxis or treatment of multidrug resistant P. falciparum malaria. Recently mefloquine resistant P. falciparum have also been reported from several endemic areas of the world. Artemisinin and its derivatives are the newer antimalarials being used for mefloquine - resistant and multidrug resistant cases of P. falciparum malaria.

Chloroquine and primaquine are the two drugs of choice for the treatment of vivax malaria. Chloroquine is given to treat the erythrocytic stages

of the parasites and primaquine to treat the persistent hypnozoite stage of parasites[54].

Prevention :

Chemoprophylaxis :

Chemoprophylaxis for travelers visiting endemic areas provides effective protection. The prophylactics of choice include chloroquine primaquine, proguanil, amodiaquine and Fansidar in weekly doses or doxycycline daily. Long-term use of antimalarial prophylactics may produce serious side effects[10,55].

Immunoprophylaxis :

A number of vaccines have been tested during last three decades. SSP-2 is currently the most likely vaccine of promise for use in falciparum malaria. The most current approach is cocktail vaccine, which makes use of combination of multiple epitopes from different malarial antigens and from different stages of parasite in its life cycle[55].

Control Measure :

Vector control is still one of the primary weapons to control malaria in endemic areas. The spraying of the indoor surface of houses with residual insecticides (DDT, Malathion, fenifrothion) is still the most effective measures to kill the adult mosquitoes. Man-vector contact can be reduced by other

preventive measures such as the use of repellents, protective clothing, bed nets (impregnated with delta methrin) and mosquito coils[56a].

Biological measures include - larvicidal fish (Gambusia) and Bio-pesticides (Bacillus thuringiensis var israelensis H14) and physical control measures are covering of over-head tanks, land filling, meshed doors and windows. Thus malaria can be controlled by effective vector control measures.

AIMS AND OBJECTIVES

i. To meet the need for a reliable diagnostic adjunct to microscopy for malaria in the clinical settings.

ii. To overcome the problem of time constraints and low sensitivity in diagnosing malaria with a low level of parasitemia by peripheral blood smear microscopy.

iii. To study the efficacy and accuracy of recent techniques to diagnose malaria.

MATERIALS AND METHODS

The present study on “Recent advances in laboratory techniques for diagnosis of malaria” was conducted in the Department of Microbiology, Stanley Medical College and Hospital during the period from May 2004 to April 2005 in collaboration with the Department of Medicine and Jawaharlal Nehru Institute of Social Paediatrics.

The study group includes :

I. 190 patients clinically diagnosed as malaria.

II. 20 patients diagnosed as Enteric fever - Disease Control

III. 20 apparently healthy individuals - Healthy Control.

Total of 230 blood samples were collected, of these 190 blood samples were collected from clinically diagnosed as malaria patients with symptoms of fever and chills irrespective of age and sex. 20 blood samples each were collected from patients diagnosed with enteric fever (disease control) and from healthy individuals (healthy control). The criteria for the selection of healthy control were the history of absence of fever for a period of one month prior to the study.

190 cases of clinically diagnosed malaria and control group n=40 (disease control, healthy control) were tested by Peripheral blood smear and Quantitative Buffy Coat (QBC). From the 190 cases 61 were selected by

simple random method and tested by recent diagnostic tests such as Immunochromatographic test for antigen detection (HRP-2 and pLDH), DOT ELISA for P. falciparum, DRDE (HRP-2 antigen) and P.C.R. assay.

Control groups (20 disease control and 20 healthy control) were subjected to Immuno chromatographic test (Now ICT), DOT ELISA for Plasmodium falciparum (DRDE) and P.C.R. assay.

Collection of Blood Samples :

Under strict aseptic precautions, thick and thin peripheral blood smears were prepared by finger prick, and 3-5 ml of blood samples were collected in a pre-sterilized aliquot with anticoagulant (EDTA) by venepuncture under strict aseptic precautions from the patients seeking treatment in in-patient and outpatient department of Government Stanley Hospital before administration of anti malarial drug.

Transport and storage of samples :

The blood samples collected from patient were transported to the laboratory immediately. Thick and Thin smears were prepared and stained by JSB technique. Q.B.C. assay was also carried out simultaneously. Remaining portion of the samples was aliquoted and stored at 4-8^{o}C for subsequent ICT, Dot ELISA for Plasmodium falciparum and PCR assay.

Methodology :

I. **Peripheral blood smear :** Thick and thin peripheral blood smears were prepared on a clean grease free glass slide.

a. **Thin smear :** Smears were air dried, fixed with methanol, dipped in JSBII stain for 2-3 sec, washed with buffer water to remove excess stain, dipped in JSB I stain for 40-50 sec and air dried.

b. **Thick smear :** The smears were dehaemoglobinized using buffer water for 5-10 minutes and continued the staining procedure as for thin smear. Both thick and thin smears were examined under oil immersion.

II. Q.B.C. Assay (Quantitative Buffy Coat)

The Q.B.C. blood parasite detection system consists of capillary tubes coated with acridine orange, floats, closures, a capillary tube holder, microcentrifuge and an ordinary light microscope with customized fluorescence – paralens attachment. The QBC capillary tube was filled with about 50-60 µl of blood soon after the collection. The blood was mixed with the precoated contents (acridine orange and potassium oxalate) by gently rotating and tilting the tube atleast five times. The cap was firmly affixed and the float inserted inside the tube. The Q.B.C. tube was then centrifuged at 12,000 rpm for 5 minutes. The tube was mounted on the capillary tube holder and examined using the paralens under 60 X oil immersion objective. The Q.B.C. tube was examined for atleast 20 Q.B.C. fields. The parasites are

concentrated in defined areas within the tube, which result in great reduction of examination times to 1-3 minutes.

The principle of Q.B.C. is based on the fact that on centrifugation at a high speed, the whole blood separates into plasma, buffy coat and packed red cell layer. The float gets buoyed by the packed blood cells and is automatically positioned within the buffy coat layer. Blood cells in the buffy coat layer separate according to their densities; platelets remaining at the top, lymphocytes and monocytes within the middle layer and granulocytes at the bottom. Due to acridine orange the malarial parasite stains green (DNA, nucleus) and orange (RNA, cytoplasm). The tube is examined in the region between the RBC and granulocytes and within the granulocytes and mononuclear cell layer where parasites are most abundant.

Immunochromatographic test (detection of HRP-2 and PMA)

Test Principle : This is a rapid in vitro immunodiagnostic test for the detection of circulating P. falciparum HRP-2 and an antigen common to all four species of malaria, pan malarial antigen (PMA) in whole blood. The test uses two specific monoclonal antibodies that have been immobilized across the test strip. One antibody is specific for the histidine-rich protein-2 antigen of P.f., (HRP-2). The other antibody is specific for an antigen that is common to all four plasmodium species.

A procedural control line is also immobilized across the test strip and

will always appear in area C of the test window, if the test has been performed correctly. 15μL of whole blood is applied to a sample pad impregnated with colloidal gold-labeled antibodies, which are directed against the malarial antigens. When a positive sample is applied, malarial antigens bind to the gold-conjugate antibodies in the sample pad. Reagent is then added which allows the immune complexes formed to migrate along the test strip where they are captured by the immobilized antibodies. When capture occurs, one or two pink lines will form in the test window. When a negative sample is applied only the control line will appear.

Test Procedure :

- All test components were brought to room temperature prior to use.
- With EDTA capillary tube blood sample was applied to cover the entire purple sample pad.
- Two drops of Reagent A was applied to the white pad immediately below where the blood was applied.
- Four drops of Reagent A was applied to the pad located at the top of the left hand side of the test card.
- Adhesive on the right hand side of the test card was removed and discarded.
- The blood sample was allowed to run up the full length of the test strip.
- The card was closed immediately.
- The result was read through the viewing window after 10 minutes.

Interpretation :

Plasmodium falciparum infection : A positive test result is indicated by any visible line in the test window next to T1 together with a line in area C.

Plasmodium falciparum infection or a mixed infection (Plasmodium falciparum,Plasmodium ovale, Plasmodium malariae, Plasmodium vivax) : A positive test result is indicated by any visible line in the test window next to T1 and T2, together with a line in area C.

Plasmodium ovale, Plasmodium malariae, Plasmodium vivax or a mixed infection of all three : A positive test result is indicated by any visible line in the test window next to T2, together with a line in area C.

Negative Test Result : The test is negative if only the C line appears.

DOT ELISA FOR P. falciparum (DRDE) :

Test Principle : Rabbit polyclonal antibodies to HRP-2 antigen are coated to the Nitrocellulose membrane. Antigen present in plasma/serum binds to this antibody. In the next step a revealing antibody with high sensitivity and specificity is added. The resulting complex is detected using enzyme substrate reaction. Appearance of brown colored dots on 2 NC tips indicates positive result and a brown dot on 1 NC tip indicates negative result.

Test Procedure :

- All the kit contents were brought to room temperature.
- The cap of 2-tip NC vial (Green cap vial) was labeled.
- 5 drops of dilution buffer were added on the NC tips in the green cap.

- 1 drop of blood sample was added in the containing dilution buffer and incubated at room temperature for 20 minutes; discarded the contents after incubation.
- Few drops of wash buffer were added in the vial cap with the help of syringe and rings. Then 1 ml of wash buffer was added in the vial; this vial was closed with cap containing NC tips and incubated with the diluted sample; shake vigorously for 20 to 30 seconds for washing the NC tips; discarded the contents.
- 1 ml of fresh wash buffer was added and the washing was repeated.
- Again the used wash buffer was discarded and the cap was dried.
- 4 drops of monoclonal antibody was added and incubated for 20 minutes at room temperature; washing was repeated as before.
- 4 drops of conjugate was added over the NC tips in the cap and incubate at room temperature for 20 minutes; washing was repeated for 3 times
- Half spoon of substrate powder was added in the vial. The vial was half filled with substrate buffer and closed with cap.
- The vial with substrate solution was shaked vigorously for 2 to 3 minutes and the appearance of colored dots on the NC tips was observed.

Interpretation :

Appearance of a colored dot only at one NC tip indicates a negative result. Appearance of colored dots in both NC tips indicates positive result for P. falciparum antigen.

PCR Amplification of target DNA sequence :

Chromosomal DNA preparation :

Chromosomal DNA was extracted from about 300 µl of whole blood with a slight modification of the standard procedure of Maniatis *et al* (1982). The red blood cells were harvested by centrifugation of the whole blood at 3000X G for 2 minutes. The cells were washed three times in cold Phosphate Buffer Solution. The pellet was resuspended in 1 ml of Phosphate Buffer Solution. About 10 µl of 5% saponin was added and gently mixed for a final concentration of 0.05%. After lysis was observed, centrifugation was done immediately at 6000X G for 5 min. To the pellet, 25 µl of lysis buffer and 75µl of distilled water was added and was incubated at 37^0C for approximately 3hours with intermittent mixing by hand. After the incubation, about 100 µl of distilled water and 200 µl of tris saturated phenol was added, mixed well and centrifuged at 12,000 rpm for 8 minutes. The supernatant was collected and extracted likewise with 200 µl of chloroform. The genomic DNA from the aqueous layer was then precipitated by adding 1/10 volume of sodium acetate and 2.5 volumes of absolute ethanol for a couple of hours or overnight at -20^0C. The precipitate was centrifuged for 30 min at 4^0C, washed gently once with 70% ethanol, dried and the pellet was gently resuspended in Tris-EDTA (TE) buffer and stored at $–20^0$C for further use.

Polymerase chain reaction

Primers specific for *P. falciparum* and P.vivax are presented in Table 1.

Table 1. Primers synthesized for detection of *P. falciparum and P.vivax*

PRIMER	PRIMER SEQUENCE	Product size
Pf 1	5’ AGA AAT AGA GTA AAA AAC AAT TTA 3’	918 bp
Pf 2	5’ GTA ACT ATT CTA GGG GAA CTA 3’	
P.v.1	5’ CCG AAT TCA GTC CCA CGT 3’	523 bp
P.v.2	5’ GCT TCG GCT TGG AAG TCC 3’	

Polymerase chain reaction was performed as per the method of Mullis and Falona (1987) and Saiki and co-workers (1988) using Qiagen Taq PCR core kit. The kit reagents of 10X PCR buffer, dNTP mix, Q buffer and primer solutions were thawed and master mixture was prepared as described in Table 2. To the appropriate volume of master mix, template DNA was added. The PCR programme was run in Biorad Gene cycler. The amplification involved initial denaturation at 94^{o}C for 5 minutes and 30 cycles of 1 minute denaturation at 94 ^{o}C, 1 minute of annealing at 55 ^{o}C, 1 minute of primer extension at 72 ^{o}C and a final extension at 72 ^{o}C for 10 minutes. To standardize the annealing temperature of the primers, a temperature gradient was run with the range from 50^{0}C to 58^{0}C. In all, 8 reactions were run at annealing temperatures of 50^{0}C, 50.6^{0}C, 51.6^{0}C, 52.9^{0}C, 55^{0}C, 56.5^{0}C, 57.4^{0}C and 58^{0}C.

Table 2. Standard PCR protocol

COMPONENT	VOLUME (L)	CONCENTRATION
10X PCR buffer	2.5	1X
Q buffer	5.0	-
dNTP mix (10mM each)	0.5	200mM of each dNTP
Primer - Pfcsp1f	0.5	0.1 – 0.5 MM
Primer - Pfcsp1r	0.5	0.1 – 0.5 MM
Taq DNA polymerase	0.25	1.25 units
Distilled water	10.75	-
Template DNA	5.0	50 ng/reaction
Total Volume	25	-

Table 3. PCR conditions

	TIME	TEMPERATURE
Initial denaturation	5 min	94^0C
3 step cycling		
Denaturation	1 min	94^0C
Annealing	1 min	55^0C
Extension	1 min	72^0C
Repeat cycle 30 times		
Final extension	10 min	72^0C

Agarose gel electrophoresis

The purity of the DNA was checked in submarine gel electrophoresis. Agarose gel (Sigma Chemicals, USA) 0.8 percent was prepared in TBE electrophoresis buffer (pH 7.5) containing 0.5 μg/ml concentration of ethidium bromide dye. The DNA samples were loaded in each of the wells of the gel

after solidification and run in the electrophoresis tank with TBE buffer in 100V along with molecular size marker. The gel was then visualized under UV irradiation.

STATISTICAL ANALYSIS

Qualitative data were given in frequencies with their percentages. QBC, ICT and PCR occurrence were compared with Gold Standard (Peripheral blood smear) and diagnostic statistics like Sensitivity, Specificity NPV and PPV were given with their estimate and 95% Confidence interval.

ROC curve were given for the diagnostic test results to represent Sensitivity and Specificity

RESULTS

A total of 190 patients clinically diagnosed as malaria were taken up for this study and 40 cases of control (disease control diagnosed as enteric fever n=20 and healthy control n=20) were included in the study from May 2004 to April 2005. Blood samples were collected from patients who attended the in-patient and outpatient departments of Medicine and Jawaharlal Nehru Institute of Social Paediatrics, Government Stanley Hospital.

190 cases were tested with peripheral blood smear and QBC. Out of this 61 samples were selected by simple random method and tested with recent techniques such as ICT, Dot ELISA for P. falciparum and PCR. Control groups (Disease control n=20 and Healthy control n=20) were also tested with Peripheral blood smear, QBC and ICT Dot ELISA for P. falciparum and PCR assay. All these new technologies were evaluated by comparing with the acceptated “gold standard” method. The results were analyzed statistically.

Table-1

Results of JSB Stained Peripheral blood smear Examination n=230

S. No.	Study Group	JSB Stained Peripheral blood smear Positive			Positive Percentage %
		Thick smear	Thin smear		
			P.v	P.f	
1.	190 cases	56	55	1	29.4
2.	20 Disease Control	-	-	-	0

3.	20 Healthy Control	-	-	-	0

In the present study out of the 190 cases 55 were positive for P. vivax and 1 case positive for P. falciparum by the thin smear examination. In thick smear 56 cases (29.4%) were positive for malarial parasite and its 95% confidence interval was 23% to 36%. Disease control & Healthy control (n=40) were negative for malarial parasite by JSB staining method.

Table-2 (a)
Results of QBC Assay n=230

S. No.	Study Group	QBC Positive	Positive Percentage %
1.	190 cases	109	57.3
2.	20 Disease control	0	0
3.	20 Healthy control	0	0

Among the total of 190 clinically diagnosed cases, QBC assay detected the presence of malarial parasite in 109 (57.3%) cases and its 95% confidence interval was 50% to 64%. Control groups were found to be QBC negative.

Table-2 (b)
Comparison between JSB stained Peripheral blood smear and QBC n=230

QBC assay	JSB Stained Peripheral blood smear		Total
	Positive	Negative	
Positive	56	53	109
Negative	0	121	121
Total	56	174	230

Table 2(b) shows the sensitivity of 100% specificity of 69.5%, PPV of

51.3% and NPV of 100% of QBC assay. By comparing the QBC assay and smear method, QBC assay shows higher positivity.

Table-3 (a)
Results of Immunochromatographic test n=101

S. No.	Study group	ICT	Positive Percentage %
1.	61 cases	32 (P.v 29, Pf 3)	52.4
2.	20 Disease control	0	0
3.	20 Healthy control	0	0

32 of the 61 cases were positive for malarial parasite (P.v. 29, P.f. 3) and percentage positivity 52.4% by ICT method and its 95% confidence interval was 40% to 65%. Control groups were found to be negative by this technique.

Table-3 (b)
Comparison between JSB stained Peripheral blood smear and Immuno chromatographic test (ICT) n=101

ICT	JSB Stained Peripheral blood smear		Total
	Positive	Negative	
Positive	20	12	32
Negative	1	68	69
Total	21	80	101

Table 3(b) shows the sensitivity of 95.2%, specificity of 85%, PPV of 62.5%and NPV of 98.5% by ICT method. In this present study, the sensitivity and specificity of ICT was higher when compared to JSB stained blood film.

Speciation of malarial parasites also obtained by ICT.

Table-4 (a)
Results of Dot ELISA for P. falciparum n=101

S. No.	Study group	DRDE DOT ELISA FOR P. falciparum
1.	61 cases	3
2.	20 Disease control	0
3.	20 Healthy control	0

Among the total 61 cases only 3 cases were positive by Dot ELISA for P. falciparum (DRDE). Control groups were negative by this technique. Dot ELISA for P. vivax was not done. So this method was not included in the comparative study with peripheral blood smear.

Table-5 (a)
Results obtained by PCR n=101

S. No.	Study group	PCR	Positive Percentage %
1.	61 cases	31 (P.v 28, Pf 3)	50.8
2.	20 Disease control	0	0
3.	20 Healthy control	0	0

31 out of 61 cases were positive for malarial parasite (P.v.28, P.f.3) by PCR technique with a percentage positivity of 50.8% and its 95% confidence interval was 38% to 63%. Control groups were negative by PCR method.

Table-5 (b)

Comparison between PCR technique and JSB stained Peripheral blood smear n=101

PCR	JSB Stained Peripheral blood smear		Total
	Positive	Negative	
Positive	19	12	31
Negative	2	68	70
Total	21	80	101

The sensitivity, specificity, PPV and NPV of PCR assay were calculated against the gold standard of Peripheral blood smear. The sensitivity of 90.5%, Specificity of 85%, PPV of 61.3% and NPV of 97.1% by PCR assay when compared to Peripheral blood smear.

Table-6
Evaluation of recent techniques in diagnosis of malaria

Test	Sensitivity	Specificity	PPV	NPV
QBC	100%	69.5%	51.3%	100%
ICT	95.2%	85%	62.5%	98.5%
PCR	90.5%	85%	61.3%	97.1%

Out of 190 cases tested with different techniques in detecting malarial parasite, the predominant species identified was P. vivax.

Table-7
Positivity of malarial parasite by various techniques

Test	No. Of sample	Positive %	P. Vivax	P. falciparum
PERIPHERAL BLOOD SMEAR	190	29.4	55	1
QBC	190	57.3	-	-
ICT	61	52.4	29	3
PCR	61	50.8	28	3
Dot Elisa for P. falciparum	61	-	-	3

Out of 61 randomly selected blood samples, 32 cases (P.v.29, P.f.3) were positive by ICT method with positive percentage of 52.4%. 31 (P.v28, P.f.3) cases were positive by PCR assay, with positive percentage of 50.8%. Dot ELISA for P. falciparum (DRDE) detected only 3 cases of P. falciparum, which were also detected by ICT and PCR. In the present study the predominant species causing malarial infection was found to be P. vivax.

DISCUSSION

The resurgence of malaria has renewed interest in developing not only preventive measures, but also rapid diagnostic techniques. Several methods have been developed to supplement and replace the conventional microscopic method. The most promising new malaria diagnostics are the Q.B.C. assay, Assay for detection of antigen HRP-2 and PMA by I.C.T., only HRP-2 by Dot ELISA for P. falciparum (DRDE) and detection of specific nucleic acid sequences (P.f. 918 bp, P.v 523bp) by P.C.R.

In the present study 190 patients clinically diagnosed as malaria were tested by peripheral blood smear and QBC assay for diagnosis of P. vivax and P. falciparum infection. The sensitivity and specificity of QBC assay was evaluated. Out of 190 samples 61 were selected by simple random method and tested with recent techniques such as ICT, Dot ELISA for P.falciparum and P.C.R. Control groups n=40 (disease control n=20, healthy control n=20) were also tested with Peripheral blood smear, QBC, ICT, Dot ELISA for P. falciparum and PCR assay.

The results obtained are discussed as follows. Romanowsky stains (Giemsas', Leishmans', Fields' and JSB) still appear superior for species identification.

In the present study malarial parasite was detected in 56 (P.v. 55, P.f. 1)

by peripheral blood smear (29.4%).

The study by Carol J. Palmer et al in Nov 2003 (9) reported 43 (20%) were positive for malarial parasites by microscopy out of 216 specimen from patients suspected of having malaria. Iqbal J. et al in 2001 (34), had observed 163 (31%) blood samples positive for malarial parasites by microscopy out of 515 patients.

The above two studies are almost in correlation with the present study.

Barman.D.et al (7) reported 28 (37%) were positive for malarial parasite by Giemsa stained peripheral blood smear out of 74 clinically suspected cases. Russ Forney.J et al had observed (65) 2051 (42.1%) were positive for malaria by Giemsa stained peripheral blood smear out of 4873 peripheral blood smears.

One of the studies done by Krishna B.V.S. et al in 2003 (46) showed 44 (3.07%) samples were positive by Leishman stained blood film examination out of 1435 blood films.

Carol J. Palmer et al in Jan 1998 (8) had reported 96 (P.v. 79, P.f. 17) were positive by Giemsa stained peripheral blood smear (48%) out of 202 patients suspected of having malaria.

A study by Chayani N. et al (12) had observed 122 (P.v. 96, P.f. 26) were positive by Giemsa stained peripheral blood smear (52.5%) out of 232 clinically suspected patients.

Pinto MJW. et al 2001, (59) reported 239 (10.5%) were positive by

Leishman stained peripheral blood smear out of 2274 patients presenting with pyrexia and atypical presentation.

In the present study two blood samples in which ICT, Dot ELISA for P. falciparum and PCR assay detected P. falciparum were found to be negative in blood smear examination. This may be explained by the fact that P. falciparum can sometimes sequester and may not be present in circulating blood.

Stained peripheral blood smear examination is simple and inexpensive. Parasite stages and species could be identified accurately. The main disadvantages are time consuming and skilled microscopist. Also it may give poor results with low parasitaemia [52, 57]. Still the staining procedures are used for screening purpose.

QBC method is based on fluorescent staining of the blood cells and parasites.

In the present study QBC assay detected malarial parasite in 109 (57.3%) cases out of 190 clinically suspected cases. This present study showed 100% sensitivity, 69.5% specificity, PPV 51.3% and NPV 100%.

Barman.D.et al in 2003 (7) reported a sensitivity of 100% and specificity of 100% out of 74 clinically suspected cases. Rickman et al in 1989 (64) reported a sensitivity of 70% and specificity of 98.4% of QBC assay in field condition. However in hospital condition it was observed that the

sensitivity of this assay was equivalent to Giemsa. Nandwani.S et al in 2003(52) has observed 100% sensitivity and 97.5% specificity for diagnosis of malaria by QBC assay, out of 80 cases selected from the malaria centre.

The later two studies are almost correlations with the present study.

PINTO M.J.W. et al in 2001 (59) had reported 328 (14.5%) were positive out of 2274 samples. Also demonstrated higher sensitivity and great rapidity of QBC assay as compared to Leishman stained thick and thin film.

The study done by Krishna BVS, Asha R Deshpande et al in 2003 (46) had observed 57 (3.97%) were positive for malarial parasite by QBC assay of the 1435 blood samples from patients with symptoms of the fever and chills. And also reported a higher sensitivity of QBC assay as well as its rapid interpretation, as compared to Leishman's stained blood film examination.

This is a definite advantage to laboratories, which have to screen a large number of blood samples. Sensitivity of QBC assay varies from 97% to 100%. The QBC assay would be ideal to supplement stained blood film in both clinical and epidemiological studies. The limiting factors for QBC assay are cost of microscope, special accessories, need for adequate training and expertise (52). The QBC tubes do not remain readable for more than a few days and hence cannot kept for record purposes (46).

The important factor of false positive impression of malarial parasite in QBC system should be considered here. In our study 8 cases of QBC positive

were negative by Peripheral blood smear, ICT and PCR. This could be Howell-Jolly bodies or artifacts such as cell debris and bacterial contamination (7,46).

One of the serological method, Immuno chromatography Test (ICT Pf/Pv) is based on the use of HRP - 2 antigen to detect P. falciparum infection and a pan malarial antigen (PMA) to detect P. vivax infection. In the present study we evaluated the performance Now ICT Malaria P.f / P.v, P.m & P.o. and Dot ELISA for P. falciparum (DRDE) is based on the use of HRP-2 antigen to detect P. falciparum only.

In the present study 32 (P.v. 29, P.f. 3) cases were positive by NOW ICT method (52.4%) out of 61 cases; showed sensitivity of 95.2% and specificity of 85% with positive and negative predictive values of 62.5% and 98.5% respectively.

Chayani. N. et al 2004 (12) reported 118 (P.v.93, P.f.25) cases were positive for malarial parasite (50.8%), by OptiMAL test.

Iqbal. J. et al in 2001 (32) had observed the pLDH assay detected 133 (P.v. 77 & P.f. 56) the cases were positive. The positive percentage was 25.7%. The sensitivity was 89% and specificity was 99.5%. Emiliana Tjitra et al in Aug 1999 (19) has observed sensitivity of 95.5% and specificity of 89.8% with PPV and NPV of 88.1% and 96.2% respectively, out of 560 symptomatic adults and children with a presumptive clinical diagnosis of malaria.

The study done by Carol J. Palmer et al in Jan 1998 (8) reported 91 (74 P.v., & 17 P.f.) were positive by OptiMAL test out of 202 patients suspected having malaria. The positive percentage was 45%. This assay had sensitivity of 94% and specificity of 100%. All the four above-mentioned studies are almost in correlation with the present study.

Carol. J. Palmer et al in Nov 2003 (9) had observed 42 (19%) were positive by OptiMAL assay in a total of 216 patients suspected having malaria. The sensitivity of the OptiMAL test 98%, its specificity was 100%, with positive & negative predictive values of 100% & 99 % respectively.

The study by Russ Forney J. et al in June 2003 (65) reported sensitivity of 87% and specificity of 87% for P. vivax and sensitivity of 100% and specificity of 93% for P. falciparum by the Parasight F+V assay. The sensitivity of the device was 100% for P. falciparum densities of more than 500 parasites / µl, with a sensitivity of 83% for parasite densities of ≤ 500/µl. For P. vivax sensitivity was 99% with parasite densities of >5000/µl 92% for parasite densities 1001 to 5000/µl, 94% for parasite densities of 501 to 1000/µl and 55% for parasite densities of 1 to 500 / µl.

The study done by Indrani KAR et al in 1998 (32) had reported 100% efficiency in detection of only P. falciparum by Parasight F test, out of 93 febrile patients. In a study done by Jamshaid Iqbal et al in Sep 2004 (35) has observed the overall sensitivity and specificity of malarial infection. However

the sensitivity of this assay decreases to <70% in parasitaemia <50/µl, out of 240 patients with P. falciparum mono infection.

Various studies mentioned above are almost in correlation with the present study.

In the present study Dot ELISA detected only 3 cases of P. falciparum for P. falciparum (DRDE), which were also detected by ICT and PCR assay, thereby indicating 100% efficiency in detection of P. falciparum cases.

We should consider the merits and demerits of ICT compared to the "gold standard". ICT assay is rapid and not labor intensive[64]. It could be a useful adjunct to blood film microscopy and might permit a reduction in the duration of hospitalization and give an early warning of treatment failure[1]. Furthermore advantage of ICT assay is speciation and can also be used to indicate drug resistant infection[60].

The demerits of ICT assay are false positivity due to persistence of HRP 2 and pLDH antigenemia after antimalarial therapy[35]. Factors that may contribute to these diverse findings include test kit storage conditions in the field inadequate adherence to the test protocol, or levels of parasitaemia below the detection limit [9].

In our study one case of P. vivax detected by Peripheral blood smear & QBC were not detected by ICT. This may be due to insufficient enzyme

production, which occurs during early malarial infection or the patient's blood sample-contained parasites at concentration below the detection level[12].

There are many published studies showing the improved sensitivity and specificity of PCR-based assay over microscopic and immunochromatographic diagnosis of malaria [43].

In the present study PCR assay detected malarial parasite in 31 (28P.v. 3Pf) with positive percentage of 50.8% out of 61 clinically suspected cases. This present study showed, sensitivity of 90.5% and specificity of 85%, PPV of 61.3% and NPV of 97.5%.

On performing PCR test by Barman. D. et al in 2003 (7) showed that PCR-Pf had a sensitivity of 64.28%, specificity of 100%, PPV of 100% NPV of 88.37%, out of 104 blood samples. Long et al (48) found the sensitivity of 94.6% at the parasitaemia level of 616/µl of blood.

Above mentioned studies are in correlation with the present study.

Kathy et al in May 2005 (43) reported 76 cases were positive by microscopy out of 358 clinically suspected patients, real-time PCR assay detected malarial parasite in 74 (97.4%) cases out of 76 smear positive case. Perandin. F. et al in Mar 2004 (57) reported that PCR assay detected malarial parasites in all 60 smear positive samples (100% sensitivity), with no detection of plasmodium in the remaining 60 smear negative blood samples (100% specificity) out of 122 clinically suspected patients.

The study done by Mathieu Rougemont et al, in Dec 2004 (50) compared with results of microscopy and real-time PCR and had a crude agreement of 86% for the detection of Plasmodium infection, out of 97 blood samples. Jamshaid Iqbal et al in Nov 1999 (36) had observed 145 (P.v. 120, P.f. 43 & 2 mixed infection) with positive percentage of 26% out of 550 clinically suspected patients. Jill M. Tham et al in May 1999 (41) showed that the results obtained by PCR were equivalent or superior to those obtained by microscopy, out of 52 patients were identified as being infected with malaria by blood film analysis and QBC analysis.

In our study 2 cases of both JSB stained Peripheral blood smear and QBC assay positive, were revealed negative by PCR assay (False negative). False negative result in PCR could be due to the failure of amplification of target DNA. The failure to amplify the target amplicons could be due to low copy number of the target sequence to the primer. Absence of target sequence could be due to degradation of the parasitic nucleic acids during the sample preparation and storage. Inaccessibility to target sequence could be due to inadequate cellular lysis. The presence of PCR inhibitors in the DNA solution could reduce the yield of the amplified products to below the detection level of ethedium bromide staining. Further more, excess of human DNA present in the sample can also inhibit the PCR reaction and can lower the sensitivity.

False positive results in PCR could also be due to carry over of parasite – DNA during sample processing or lower sensitivity of the designed primer

and PCR method itself. (7)

PCR has enabled wide-ranging investigations of antimalarial drug resistance polymorphisms and polymorphic antigen based strain diversity in the context of malarial vaccine trails. The limit of PCR-based detection of Plasmodium from whole blood is approximately 1 to 5 parasitized erythrocytes/ $5x10^6$ erythrocytes / µl (0.0001% parasitaemia)[17].

Real time PCR with melting curve analysis could be a rapid and objective supplement to the examination of Giemsa stained blood smears and may replace microscopy following further validation (43).

Despite the advantages of PCR, it is unlikely to be useful outside of well-equipped laboratories where a reliable source of electricity and expensive equipment are not available. These limitations exclude PCR from consideration as a field – ready rapid diagnostic test for malaria[17].

Unfortunately, conventional PCR assay are technically demanding and time-consuming. More over, they are prone to carryover contamination during the manipulation of post amplification products[50].

Laboratory methods that require more than 1 hour to provide a clear diagnosis of malaria are not considered rapid tests. Therefore, PCR cannot strictly be considered a rapid technique for the initial diagnosis of malaria at present. Currently such procedures have been established as a second line diagnosis for patients with high clinical suspicion of malaria but a negative

microscopy or when difficulty in species identification occurs [50].

In the present study the predominant species identified was P. vivax Government Stanley Hospital is located in North Chennai. In North Chennai 12,239 cases were diagnosed as malaria by microscopy during 2004. Out of this 11,861 cases were vivax malaria and 378 cases were falciparum malaria. During 2005 malaria positive cases were 10,666. Out of this 10417 were P. vivax and 249 were P. falciparum (Courtesy of Chennai Corporation).

SUMMARY

In the present study 190 blood samples from patients with symptoms of fever and chills (clinically diagnosed as malaria), 20 blood samples from enteric fever cases (disease control) and 20 blood samples from healthy individuals were collected and subjected to various techniques in detecting malarial parasites.

All the 190 cases were tested by JSB stained Peripheral blood smear and Q.B.C. assay. Malarial parasite was detected in 56 (P.v. 55, P.f 1) cases by JSB stained Peripheral blood smear (29.4%). 109 cases were found to be positive by Q.B.C. assay (57.3%). Control groups (Disease control & Healthy control) were also tested by Peripheral blood smear, QBC, ICT, DOT ELISA for P. falciparum and PCR assay. None of control samples were positive for malarial parasite.

Among the 190 cases, 61 blood samples were selected by simple random method and tested by recent diagnostic tests such as ICT, Dot ELISA for P. falciparum and PCR and compared with the “gold standard” JSB stained Peripheral blood smear.

Out of 61 randomly selected blood samples 32 cases (P.v. 29, P.f. 3) were positive by ICT method with positive percentage of 52.4%.

31 (P.v 28, P.f. 3) cases were positive by PCR assay with positive percentage of 50.8%. Dot ELISA for P. falciparum (DRDE) detected only 3 cases of P. falciparum, which were also detected by ICT and PCR. In the present study the predominant species causing malarial infection was found to be P. vivax.

In our study Peripheral blood smear is the simple and inexpensive test for detection of malarial parasite, and the QBC assay was found to be more sensitive. The recent techniques of I.C.T., DOT ELISA for P. falciparum (DRDE) and PCR assay are found to be more sensitive, specific and accurate enough to identify the Plasmodium species.

CONCLUSION

1. 190 clinically diagnosed cases of malaria were tested by JSB stained Peripheral blood smear and QBC assay.
2. 29.4% were positive by JSB stained Peripheral blood smear
3. 57.3% were QBC positive
4. Out of this 61 blood samples were selected by simple random method and tested by ICT, DOT ELISA for P. falciparum (DRDE) and PCR assay.
5. 52.4% were positive by antigen detection assay (HRP-2, PMA) by ICT P.f/ P.v method.
6. 50.8% were PCR positive.
7. 3 cases were positive by DOT ELISA for P. falciparum (DRDE).
8. P. vivax was the predominant species causing malarial infection.

Malaria rapid diagnostic devices (MRDD) have been developed with the hope that they would offer accurate, reliable, rapid, cost effective and easily available alternatives to traditional methods of malaria diagnosis. In future MRDDs will play an increasing role, where reliable microscopy is frequently poor. The new generation of non-microscopic immunochromatographic assay offers a practical chance to move the diagnosis of malaria away from the laboratory and nearer to the patient.

New rapid non-microscopy methods for the diagnosis of malaria that complement or support microscopy of blood films would be of great use in the early diagnosis and treatment of patients with malaria and in epidemiological studies.

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PROFORMA

Case study No. :

I.P. No. / O.P. No. :

Name :

Age / Sex :

Address :

Socioeconomic group :

Occupation :

Present H/o :

- Fever : Duration
  Periodicity
- Rigor
- Head ache
- Seizure
- Vomitus
- Treatment taken

On examination :

- Temperature
- Pulse
- B.P.
- Pallor
- Icterus
- Sensorium
  Orientation to time, Place and to person,
- Liver size
- Spleen size

Past H/o. : Previous attack of malaria and treatment.

Investigation :

- Peripheral smear (thick and thin)
- Q.B.C.
- ICT
- ELISA

- PCR

APPENDIX

I. Jaswant singh – Bhattacharjee stain (JSB stain)

JSB I : Methylene blue - 0.5 gm
1% sulphuric acid - 3CC
Potassium dichromate - 0.5 gm
Disodium hydrogen phosphate dihydrate - 3.5 gm
Distilled water - 500 CC

JSB II : Eosin yellow - 1 gm
Distilled water - 500 CC

Buffer water : Water - 1000 CC
Disodium hydrogen phosphate - 0.22 g
Potassium acid phosphate - 0.74 gm

Procedure :

Thin smear preparation – After air drying, fix in methanol dip in stain JSB II (2-3 sec) ; wash with buffer water to remove excess stain. Dip in stain

JSB I (40-60 sec) ; Dry the smear and observe under oil immersion.

Thick smear : Dehaemoglobinization using buffer water for 5-10 minutes. Continue as for thin smear.

Kit Contents for ICT :

Individually packaged test cards.

Capillary tubes.

Malaria Reagent A.

Product insert.

Procedure card.

Kit contents of Dot ELISA for P. falciparum (DRDE) :

- Two-tip nitrocellulose vials
- Dilution buffer
- Conjugate
- Substrate powder
- Substrate buffer
- Wash buffer
- Monoclonal antibody
- Metal Spoon
- Safety pin
- Syringe

ABBREVIATION

DRDE	-	Defence Research and Development Establishment
ELISA	-	Enzyme Linked Immuno-Sorbent Assay
HRP	-	Histidine Rich Protein
ICT	-	Immuno Chromatographic Test
JSB	-	Jaswant Singh Bhattacharjee
MRDD	-	Malaria Rapid Diagnostic Device
NPV	-	Negative Predictive Value
PCR	-	Polymerase Chain Reaction
pLDH	-	Parasite Lactate Dehydrogenase
PPV	-	Positive Predictive Value
QBC	-	Quantitative Buffy Coat
ROC	-	Receiver Operating Curve